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Phosphorylation of Yeast Hexokinase 2 Regulates Its Nucleocytoplasmic Shuttling*

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Paula Fernández-García[1], **Rafael Peláez**[2], **Pilar Herrero, and Fernando Moreno**[3]

From the Department of Biochemistry and Molecular Biology, University of Oviedo, 33006 Oviedo, Spain

Background: Nucleocytoplasmic transport of Hxk2 is mediated by the α/β-importins (Kap60/Kap95) and the Xpo1(Crm1) exportin.
Results: Hxk2 phosphorylation affects its subcellular localization and its interaction with karyopherins Kap60 and Xpo1.
Conclusion: The shuttling back and forth of Hxk2 between the nucleus and the cytoplasm is regulated by phosphorylation and dephosphorylation of serine 14.
Significance: Regulating the subcellular distribution of Hxk2 represents a novel physiological function mediated by Snf1 and Glc7-Reg1 proteins.

Nucleocytoplasmic shuttling of Hxk2 induced by glucose levels has been reported recently. Here we present evidence that indicates that Hxk2 nucleocytoplasmic traffic is regulated by phosphorylation and dephosphorylation at serine 14. Moreover, we identified the protein kinase Snf1 and the protein phosphatase Glc7-Reg1 as novel regulatory partners for the nucleocytoplasmic shuttling of Hxk2. Functional studies revealed that, in contrast to the wild-type protein, the dephosphorylation-mimicking mutant of Hxk2 retains its nuclear localization in low glucose conditions, and the phosphomimetic mutant of Hxk2 retains its cytoplasmic localization in high glucose conditions. Interaction experiments of Hxk2 with Kap60 and Xpo1 indicated that nuclear import of the S14D mutant of Hxk2 is severely decreased but that the export is significantly enhanced. Conversely, nuclear import of the S14A mutant of Hxk2 was significantly enhanced, although the export was severely decreased. The interaction of Hxk2 with Kap60 and Xpo1 was found to occur in the dephosphorylated and phosphorylated states of the protein, respectively. In addition, we found that Hxk2 is a substrate for Snf1. Mutational analysis indicated that serine 14 is a major *in vitro* and *in vivo* phosphorylation site for Snf1. We also provide evidence that dephosphorylation of Hxk2 at serine 14 is a protein phosphatase Glc7-Reg1-dependent process. Taken together, this study establishes a functional link between Hxk2, Reg1, and Snf1 signaling, which involves the regulation of Hxk2 nucleocytoplasmic shuttling by phosphorylation-dephosphorylation of serine 14.

All living organisms operate a variety of metabolic pathways that enable them to be self-sustainable. With regard to maintaining the balance of metabolite and energy levels, organisms have developed sophisticated sensing and signaling mechanisms that underlie the physiological responses to cell metabolite fluctuations. In several organisms, glucose is more than a nutrient because it has a regulatory role in important cellular processes, and hexokinase acts as an evolutionarily conserved glucose sensor (1, 2). In *Saccharomyces cerevisiae* hexokinase 2 (Hxk2)[4] is the predominant glucose kinase in cells growing in high glucose conditions (3) and has dual functions. It is a glycolytic enzyme in the cytoplasm but also acts as a regulator of gene transcription by modulating the expression of several Mig1-regulated genes in the nucleus (4–6). Fourteen percent of total Hxk2 protein was found in the nuclear fraction of a wild-type strain where it participates in regulatory DNA-protein complexes necessary for glucose repression of *SUC2*, *HXK1*, and *GLK1* genes (6–8). Hxk2 mediates its catalytic and regulatory functions through different protein domains because separation-of-function mutants convert Hxk2 from a bifunctional protein to a single function protein with activity as a mediating factor in transcription or as an enzyme with hexose phosphorylating activity (9).

In *S. cerevisiae*, Hxk2 is shuttled in and out of the nucleus to carry out its regulatory function (4, 8). The nucleocytoplasmic distribution of Hxk2 is affected by glucose availability and the transcriptional repressor Mig1. In high glucose conditions, Hxk2 translocation into the nucleus and interaction with Mig1 are mediated by a 10-amino acid motif of Hxk2 located between Lys-6 and Met-15 and the serine 311 of Mig1 (4, 5, 10). Nuclear Hxk2 interacts with Mig1 and Snf1 to generate a repressor complex, thereby preventing the Snf1 protein kinase-mediated phosphorylation of Mig1 at serine 311. In low glucose, Hxk2 is phosphorylated *in vivo* at Ser-14 (11) by an unknown kinase (Hxk2 is numbered from residues 1 to 485; residue 1 is a valine because the initiator methionine is cleaved off of the primary translation product), and it was suggested that the phosphoryl-

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[2] Supported by a Severo Ochoa fellowship from Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnología Foundation (Principado de Asturias). Present address: Centro de Investigación Médica Aplicada, Universidad de Navarra, 31008 Pamplona, Spain.
[3] To whom correspondence should be addressed: Dept. de Bioquímica y Biología Molecular, Universidad de Oviedo, Campus de "El Cristo," 33006 Oviedo, Spain. Tel.: 34-985-103-567; Fax: 34-985-103-157; E-mail: fmoreno@uniovi.es.

[4] The abbreviations used are: Hxk2, hexokinase 2; NES, nuclear export signal; NLS, nuclear localization sequence.

ation state of this amino acid affects glucose signaling (7, 12) and the Hxk2 nuclear export process (13). The phosphorylated Hxk2 loses the interaction with Mig1 and leaves the complex, and serine 311 of Mig1 is phosphorylated by the Snf1 protein kinase. Under these conditions, the repressor complex is disorganized, and phosphorylated Hxk2 and Mig1 leave the nucleus (4, 5). Thus, Snf1 protein kinase, a member of the Snf1/AMP-activated protein kinase family, has an essential role in derepression of Mig1-Hxk2-repressed genes (14). The kinase is a heterotrimeric complex comprising a catalytic subunit, α (Snf1), and two regulatory subunits, β (Sip1, Sip2, or Gal83) and γ (Snf4) (15). Snf1 activity requires phosphorylation of threonine 210 in the catalytic subunit by one of the three protein kinases, Sak1, Tos3, or Elm1 (16–18). ADP appears to play a major role in Snf1 activation in response to glucose limitation by protecting the enzyme against dephosphorylation by the Glc7-Reg1 protein phosphatase (19).

To carry out its functions, Hxk2 has to shuttle in and out of the nucleus, but the protein is too large to translocate through the nuclear pore complex by diffusion; therefore, Hxk2 transport across the nuclear envelope must be a mediated and regulated process. Recently, the carrier proteins involved have been described. The mechanism by which Hxk2 enters the nucleus is mediated by the α/β-importin (Kap60/Kap95) pathway (20). The Hxk2 nuclear import and the binding of Hxk2 with Kap60 are glucose-dependent processes and involve one lysine-rich NLS located between Lys-6 and Lys-12. Moreover, Kap95 facilitates the recognition of the Hxk2 NLS motif by Kap60, and both importins are essential for Hxk2 nuclear import. The Hxk2 nuclear export and the binding of Hxk2 and Xpo1 are regulated by glucose levels and involve two leucine-rich NESs located between Leu-23 and Ile-33 (NES1) and Leu-310 and Leu-318 (NES2). Thus, the mechanism by which Hxk2 leaves the nucleus is Xpo1 (Crm1)-dependent (13). Nucleocytoplasmic shuttling has been described for Hxk2, but the mechanism of its regulation has not been elucidated. Because intramolecular masking of NLS or NES domains of the protein cargoes by dimerization or phosphorylation is probably the most widespread mechanism of nucleocytoplasmic transport regulation (21–24) and serine 14 of Hxk2 is phosphorylated *in vivo* under low glucose growth conditions (11), it was hypothesized that the phosphorylation state of this amino acid could affect the nucleocytoplasmic distribution of Hxk2.

In this study, we describe how interactions of Kap60 and Xpo1 with Hxk2 are regulated by the phosphorylation state of serine 14. Fluorescence microscopy experiments demonstrated that the association of Hxk2 with Kap60 and Xpo1 correlates with the nucleocytoplasmic distribution of Hxk2. Bioinformatics structural analysis showed that Hxk2 phosphorylation at serine 14 creates an acidic patch in the NLS motif that is important for the nuclear export. As predicted, we found that Hxk2 is a substrate for Snf1 protein kinase both *in vivo* and *in vitro*. Subsequent studies demonstrated that Glc7-Reg1 is the protein phosphatase involved in the dephosphorylation process. These results provide an important role for both Snf1 and Glc7-Reg1 in regulating Hxk2 activity in the nucleus as well as gene transcription by modulating the expression of several Mig1-regulated genes.

TABLE 1
***S. cerevisiae* strains used in this study**

Name	Relevant genotype	Source/Ref.
W303-1A	*Matα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100*	25
DBY1315	*Matα ura3-52 leu2-3,2-112 lys2-801 gal2*	26
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
DBY2052	*Matα hxk1::LEU2 hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2*	26
H250	*MATα SUC2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-δ2::LEU2*	7
FMY303	*Matα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 snf1::SNF1-HA*	5
FMY308	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk2::HXK2S14A*	This work
FMY309	*MATα ura3-52 leu2-3,2-112 lys2-801 gal2 hxk2::HXK2S14D*	This work
FMY310	*Matα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 snf1::SNF1-HA mig1-δ2::LEU2*	This work
MB12	*Matα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 tpk1::LEU2 tpk2::HIS3*	59
MB13	*Matα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 tpk1::LEU2 tpk3::URA3*	59
MB23	*Matα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 tpk2::HIS3 tpk3::URA3*	59
CJM492	*Matα ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*	47
Y01261	*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; tpk1::kanMX4*	Euroscarf
Y11089	*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; tpk2::kanMX4*	Euroscarf
Y15016	*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; tpk3::kanMX4*	Euroscarf
Y14311	*MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; snf1::kanMX4*	Euroscarf
Y10878	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; tda1::kanMX4*	Euroscarf
Y13967	*Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; reg1::kanMX4*	Euroscarf

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The *S. cerevisiae* strains used throughout this study were derived from W303-1A (25), DBY1315 (26), and BY4741 (Euroscarf) haploid wild-type strains and are listed in Table 1.

We created two mutant alleles of *HXK2* by mutating the *HXK2* gene in YEp352-HXK2 plasmid to generate *HXK2(S14A)* (encodes Hxk2S14A protein) and *HXK2(S14D)* (encodes Hxk2S14D protein) genes using a PCR-based mutagenesis protocol. The Kan^R gene was inserted into the XbaI site located in the yeast genome 371 bp after the *HXK2* stop codon of plasmids YEp352/Hxk2S14A and YEp352/Hxk2S14D. Then by using these constructs, recombination cassettes were obtained by PCR. These cassettes contain the mutated *HXK2* locus, the Kan^R marker, and 515 bp from the 5′-genomic region downstream of the *HXK2* gene. These linear DNAs were integrated into the *HXK2* locus of strain W303-1A. To confirm the correct insertion, we PCR-amplified the *HXK2* locus from strains

FMY308, which contains the *HXK2* gene replaced by the *HXK2(S14A)* gene, and FMY309, which contains the *HXK2* gene replaced by the *HXK2(S14D)* gene, to detect the presence of the *HXK2(S14A)* and *HXK2(S14D)* mutant alleles, respectively, by sequencing analysis. The FMY303 strain was created by homologous recombination with the 3HA-T_{ADH1}-kanMX6 cassette as indicated previously (5). The FMY310 strain was created by homologous recombination with the *mig1-δ2::LEU2* cassette obtained by PCR from the H250 strain (27). *Escherichia coli* DH5α (*F Ø80dlacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17(rk-rk-) supE44 relA1 deoRΔ 99U169*) was the host bacterial strain for the recombinant plasmid constructions.

Yeast cells were grown in the following media: YEPD, high glucose (2% glucose, 2% peptone, and 1% yeast extract), YEPGly, low glucose (0.05% glucose, 3% glycerol, 2% peptone, and 1% yeast extract), and synthetic media containing the appropriate carbon source and lacking appropriate supplements to maintain selection for plasmids (2% glucose (SD) or 3% glycerol and 0.05% glucose (SGly) and 0.67% yeast nitrogen base without amino acids). Amino acids and other growth requirements were added at a final concentration of 20–150 μg/ml. The solid media contained 2% agar in addition to the components described above.

Plasmids—The yeast expression plasmids YEp352/Hxk2-GFP and YEp352/Hxk2*nes2*-GFP were constructed as indicated previously (4, 13). To create Hxk2 mutant alleles Hxk2S14A-GFP and Hxk2*nes2*S14D-GFP, the plasmids YEp352/Hxk2-GFP and YEp352/Hxk2*nes2*-GFP were used as templates in conjunction with oligonucleotides HXK2S15A-d (AAAACCACAAGCCAGAAAGGGTGCCATGGCCGATGTGCCAAAGGAATTGA), HXK2S15A-r (TCAATTCCTTTGGCACATCGGCCATGGCACCCTTTCTGGCTTGTGGTTTT), HXK2S15D-d (AAAACCACAAGCCAGAAAGGGTGACATGGCCGATGTGCCAAAGGAATTGA), and HXK2S15D-r (TCAATTCCTTTGGCACATCGGCCATGTCACCCTTTCTGGCTTGTGGTTTT) in the PCR-based site-directed mutagenesis method (28). All nucleotide changes were verified by DNA sequencing. Plasmid FMp111/Hxk2-GFP for expression of Hxk2-GFP in yeast was constructed by subcloning the *ADE2* gene under the control of its own promoter in the BamHI site of YEp352/Hxk2-GFP plasmid. Plasmids pWS93/Snf1-HA and pWS93/Snf1T210A-HA for expression of Snf1 and Snf1T210A HA-tagged proteins in yeast were a gift from the laboratory of P. Sanz (29, 30).

GST fusion vector pGEX/Hxk2 was constructed as indicated (4). Plasmid pYEX/Snf1 for expression of GST-Snf1 in yeast was a gift from the laboratory of D. J. Thiele (31), and plasmids pGEX/Kap60 and pGEX/Xpo1 for expression of GST-Kap60 (32) and GST-Xpo1 (33) in *E. coli* were gifts from the laboratories of M. P. Rout and C. N. Cole.

Fluorescence Microscopy—Yeast strains expressing the Hxk2-GFP, Hxk2S14A-GFP, or Hxk2*nes2*S14D-GFP were grown to early log phase (A_{600} of less than 0.8) in synthetic high glucose medium (SD-Ura). Half of the culture was shifted to synthetic low glucose medium (SGly-Ura) for 1 h. The media contained the appropriate carbon source and lacked the appropriate supplements to maintain selection of plasmids. Cells (25 μl) were loaded onto poly-L-lysine-coated slides, and the remaining suspension was immediately withdrawn by aspiration. One microliter of DAPI (2.5 μg/ml in 80% glycerol) was added, and a cover slide was placed over the microscope slide. GFP and DAPI localization in live cultures was monitored by direct fluorescence using a Leica DM5000B microscope. To avoid the nonlinear range of fluorescence signals, cells highly overexpressing GFP-tagged fusion protein were excluded from further analyses. The localization of proteins was monitored by visual inspection of the images. At least 100 cells were scored in each of at least three independent experiments. Images representative of the results obtained are shown. Images were processed in Adobe Photoshop CS.

Statistical Analysis—Data are the result from at least three independent experiments. Results are shown as the mean ± S.E. Statistical analysis was performed using SPSS 15.0. A normal distribution was assured with the Kolmogorov-Smirnov test, and data were compared with Student's *t* test for two independent samples. Significant differences were considered when $p < 0.01$.

Protein Structural Models—The full-length Hxk2 protein has not been crystallized. In the best approach, the protein lacks 17 amino acids of its N-terminal region containing the NLS motif and is composed of two lobes with a cleft in the middle that coincides with the active site of the enzyme (Protein Data Bank code 1IG8) (34). To get a structure prediction of the NLS region, we used the I-TASSER server and the amino acid sequence of the *S. cerevisiae* Hxk2. The server uses sequence alignments of polypeptides of known structure to form, from the amino acid sequence of the protein, the protein structure more probable and close to the actual structure (35, 36). To measure the distances between the Cα of different amino acids within a model and for protein hydrophobicity imaging, Jmol and Protein Workshop programs, respectively, were used.

Preparation of Crude Protein Extracts—Yeast protein extracts were prepared as follows. Yeast were grown in 10–20 ml of YEPD or synthetic high glucose medium (SD-Ura) at 28 °C to an optical density at 600 nm of 0.8–1.0. Half of the culture was shifted to YEPGly or synthetic low glucose medium (SGly-Ura) for 1 h. Cells were collected, washed twice with 1 ml of 1 M sorbitol, and resuspended in 500 μl of PBS buffer (150 mM NaCl, 100 mM Na_2HPO_4, 18 mM NaH_2PO_4, pH 7.3). The cells were broken in the presence of glass beads by one pulse of 20 s at 6.0 m/s using a FastPrep homogenizer (Thermo Electron Co.). After centrifugation at 17,000 × *g* for 15 min at 4 °C, the supernatant was used as crude protein extract. After centrifugation at 6,100 × *g* for 15 min at 4 °C, the supernatant was used as crude protein extract in immunoprecipitation and GST pull-down experiments.

Immunoblot Analysis—Mutant or wild-type yeast cells were grown to an optical density at 600 nm of 0.8–1.0 in selective medium containing high glucose (2%) and shifted to low glucose conditions for 1 h. The cells were collected by centrifugation (3,000 × *g* at 4 °C for 2 min), and crude extracts were prepared as described above. For Western blotting, 20–40 μg of proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to enhanced chemiluminescence PVDF transfer membrane (Amersham Biosciences Hybond™-P, GE Healthcare) by electroblotting. The

membrane was then incubated with anti-Hxk2, anti-Kap60 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-HA (Cell Signaling Technology) as primary antibody followed by the appropriate secondary antibody. Horseradish peroxidase-conjugated protein A was used as secondary reactant. The SuperSignal West Pico Chemiluminescent system (Pierce) was used for detection.

Co-immunoprecipitation Assay—Immunoprecipitation experiments were performed using whole cell extracts from different strains. The extracts were incubated with anti-Kap60, anti-Hxk2, or anti-Pho4 polyclonal antibody for 3 h at 4 °C. Protein A-Sepharose beads (GE Healthcare) were then added and incubated for 3 h at 4 °C in a spinning wheel. After extensive washing with PBS buffer, immunoprecipitated samples were boiled in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromphenol blue, 10% glycerol). The supernatant was subjected to 12% SDS-PAGE. The proteins were transferred to an enhanced chemiluminescence PVDF membrane and immunoblotted as described above using anti-Hxk2 or anti-HA monoclonal antibody. Values shown are representative results from individual experiments.

GST Pulldown Experiments—E. coli cells from the BL21(DE3) pLysS strain were transformed with the fusion protein expression vector pGEX/Kap60 or pGEX/Xpo1. Cells were grown to an $A_{600\,nm}$ of 0.5–0.8, induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 3 h, and collected by centrifugation. The cell pellet was resuspended in PBS buffer and sonicated. Insoluble material was removed by centrifugation (17,000 × *g* for 20 min at 4 °C). Soluble extract was incubated with glutathione-Sepharose 4B beads (GE Healthcare) for 1 h at 4 °C, washed extensively with PBS buffer, and resuspended in the same buffer. The GST-Kap60 and GST-Xpo1 fusion proteins coupled to glutathione-Sepharose were incubated with yeast whole cell extracts from the wild-type and mutant yeast strains expressing Hxk2, Hxk2S14A, or Hxk2S14D proteins, respectively, for 1 h at 4 °C in PBS buffer. The cell extracts were obtained from yeast cells grown in YEPD medium containing high glucose (2%) and shifted to 0.05% glucose plus 3% glycerol (low glucose) for 1 h. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 μl of sample loading buffer, and analyzed by SDS-PAGE followed by Western blotting using anti-Hxk2 antibodies and horseradish peroxidase-conjugated protein A. Bound antibodies were detected using the SuperSignal West Pico Chemiluminescent system (Pierce).

Yeast cells from the W303-1A strain were transformed with the expression vector pYES/Snf1. Cells were grown to an $A_{600\,nm}$ of 1.0, induced with 0.5 mM $CuSO_4$ at 28 °C for 1 h, and collected by centrifugation. The cell pellet was broken in the presence of glass beads by one pulse of 20 s at 6.0 m/s using a FastPrep homogenizer (Thermo Electron Co.). After centrifugation at 17,000 × *g* for 15 min at 4 °C, the supernatant was used as crude protein extract for *in vitro* experiments.

In Vitro Kinase Assay—In the Snf1 *in vitro* kinase assay, we used Snf1 purified from yeast cells transformed with the expression plasmid pYEX/Snf1 and Hxk2 purified from bacteria transformed with the expression plasmid pGEX/Hxk2. The GST-Snf1 and GST-Hxk2 fusion proteins coupled to glutathione-Sepharose beads were incubated with 2.5 units of thrombin (2 h at 4 °C) for site-specific separation of the GST affinity tag from Snf1 and Hxk2 proteins. Identical amounts of Snf1 and Hxk2 affinity-purified proteins were immediately incubated for 30 min at 30 °C in the presence of 2.2 mM ATP, 0.5 mM EDTA, 0.5 mM DTT, and 5 mM magnesium acetate. The GST-Xpo1 fusion protein coupled to glutathione-Sepharose was incubated with the assay mixture for 1 h at 4 °C in PBS buffer. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 μl of sample loading buffer, and analyzed by SDS-PAGE followed by Western blotting using anti-Hxk2 or anti-Ser(P) (Cell Signaling Technology) antibody and horseradish peroxidase-conjugated protein A. Bound antibodies were detected using the SuperSignal West Pico Chemiluminescent system (Pierce).

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously (37). Cells were harvested and disrupted by vortexing in the presence of glass beads, and the lysate was sonicated to generate DNA fragments that ranged in size from 200 to 400 bp. To immunoprecipitate HA-tagged proteins, we incubated the extract overnight at 4 °C with anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To immunoprecipitate Hxk2 protein, we incubated the extract overnight at 4 °C with anti-Hxk2 antibodies (8). The primer sequences for PCR to amplify the *SUC2* promoter region containing the MIG1 element were 5′-TTATTACTCTGAACAGGA-3′ (sense) and 5′-AAGTCGTCAAATCTTTCT-3′ (antisense).

RESULTS

Hxk2 Phosphorylation Affects Its Interaction with the Karyopherins Kap60 and Xpo1—Hxk2 is found both in the nucleus and in the cytoplasm of *S. cerevisiae*; nuclear localization is dependent on the presence of an NLS located between lysine 6 and lysine 12. Although serine 14, which resides near the NLS, can be phosphorylated in the absence of glucose (11), it is still unknown whether this phosphorylation plays a role in the affinity of the α-importin (Kap60) for the NLS and of the exportin Xpo1 for the NES of Hxk2. Because in low glucose conditions Hxk2 is phosphorylated at serine 14 and is predominantly cytoplasmic (4, 11), we hypothesized that phosphorylation of this residue blocks the nuclear import and enhances the nuclear export of Hxk2. To assess this, Hxk2 serine 14 was mutated by site-directed mutagenesis to non-phosphorylatable alanine (Hxk2S14A) or phosphomimetic aspartic acid (Hxk2S14D), and we examined the effect on Hxk2 variant interaction with both Kap60 and Xpo1 karyopherins at high and low glucose conditions.

In accordance with previous results (20), the interaction between wild-type Hxk2 and Kap60 was stronger with samples from high glucose-grown cultures, indicating that the high glucose condition increases the affinity of Hxk2 for the import receptor (Fig. 1*A*, *lanes 3* and *4*). However, the mutant mimetic form with a non-phosphorylatable Hxk2 (Hxk2S14A) is devoid of glucose-dependent regulation of its interaction with Kap60. As shown in Fig. 1*A*, a strong and specific signal was observed in both high and low glucose conditions with samples immunoprecipitated with anti-Kap60 antibody in the strain expressing the Hxk2S14A mutant protein, indicating that the serine 14 to alanine mutation increases the affinity of Hxk2 for the Kap60

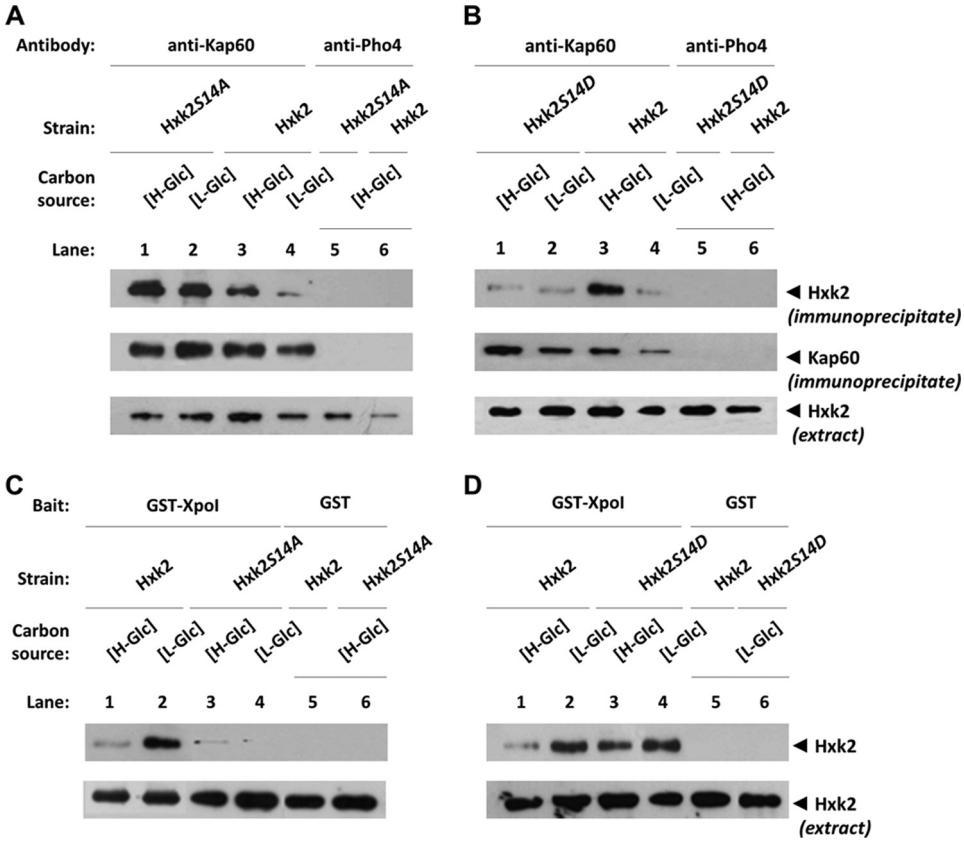

FIGURE 1. **Interaction of Kap60 and Xpo1 with Hxk2 variants.** *A* and *B*, *in vivo* co-immunoprecipitation of Kap60 with Hxk2, Hxk2S14A, and Hxk2S14D is shown. The W303-1A (WT), FMY308, and FMY309 strains were grown in YEPD medium until an $A_{600\ nm}$ of 0.8 was reached and then shifted to high (*H-Glc*) and low (*L-Glc*) glucose conditions for 1 h. The cell extracts were immunoprecipitated with a polyclonal anti-Kap60 antibody (*lanes 1–4*) or a polyclonal antibody to Pho4 (*lanes 5* and *6*). Immunoprecipitates were separated by 12% SDS-PAGE, and co-precipitated Hxk2 variants were visualized on a Western blot with monoclonal anti-Hxk2 antibody. The level of immunoprecipitated Kap60 in the blotted samples was determined by using anti-Kap60 antibody. The level of Hxk2 present in the different extracts was determined by Western blot using anti-Hxk2 antibody. *C* and *D*, GST pulldown assay of the interaction between Xpo1 and Hxk2 variants. The GST-Xpo1 fusion protein was purified using glutathione-Sepharose columns. Equal amounts of GST-Xpo1 were incubated with cell extracts from the W303-1A (WT), FMY308, and FMY309 strains. The yeast strains were grown in YEPD medium until an $A_{600\ nm}$ of 0.8 was reached and then shifted to low glucose conditions for 1 h. After exhaustive washing, the proteins were separated by 12% SDS-PAGE, and retained Hxk2 variants were visualized on a Western blot with polyclonal anti-Hxk2 antibody (*lanes 1–4*). For the control samples, GST protein was also incubated with the high and low glucose cell extracts, respectively, but no signals were detected (*lanes 5* and *6*). The level of Hxk2 present in the different extracts used in *C* and *D* was determined by Western blot using anti-Hxk2 antibody. All Western blots shown are representative of results obtained from four independent experiments.

protein under low glucose conditions. This result prompted us to investigate whether the phosphomimetic Hxk2 mutant form (Hxk2S14D) is also devoid of glucose-dependent regulation of its interaction with Kap60. To check this possibility, we inspected the interaction between Hxk2S14D and Kap60 in both high and low glucose conditions by immunoprecipitation assays. As shown in Fig. 1*B*, the signal observed under high glucose conditions with the Hxk2S14D was much weaker than that observed with wild-type Hxk2. Moreover, similar amounts of Kap60 protein were detected in the immunoprecipitate when anti-Kap60 antibody was used. Similar amounts of the Hxk2 protein variants were also detected in different protein extracts used in immunoblot analysis with an anti-Hxk2 antibody (Fig. 1, *A* and *B*). When an anti-Pho4 antibody was used to detect unspecific immunoprecipitation, no signals were observed.

To verify whether the Xpo1 interaction could also be affected by using phosphomimetic and non-phosphorylatable forms of Hxk2, *in vitro* binding assays were performed using recombinant proteins. We used crude protein extracts from Hxk2-, Hxk2S14A-, and Hxk2S14D-producing strains and bacterially produced GST-Xpo1 fusion protein. As shown in Fig. 1*C*, a clear retention of the Hxk2 protein was observed for the sample containing GST-Xpo1 and crude extract from the strain expressing the wild-type Hxk2 protein under low glucose conditions. No retention or a very weak retention was detected with extracts obtained from the same strain under high glucose conditions. These results confirm that the Hxk2 protein interacts with Xpo1 in a glucose-dependent manner. However, when we performed a similar interaction experiment with samples containing GST-Xpo1 and crude extracts from strains expressing Hxk2S14A (Fig. 1*C*) and Hxk2S14D (Fig. 1*D*), the glucose-dependent regulation was lost. As shown in Fig. 1*C*, a very weak retention of Hxk2S14A was observed under low glucose conditions, suggesting that Xpo1 interacts preferentially with phosphorylated Hxk2. Moreover, a strong and specific retention of Hxk2S14D was observed under high glucose conditions, indicating that Xpo1 interacts with the phosphomimetic Hxk2 both at high and low glucose conditions (Fig. 1*D*). When a control with the GST protein in the reaction mixture was used, no signal of Hxk2, Hxk2S14A, or Hxk2S14D was observed. Taken together, the results from the interactions of different Hxk2 variants with Kap60 and Xpo1 suggest that both

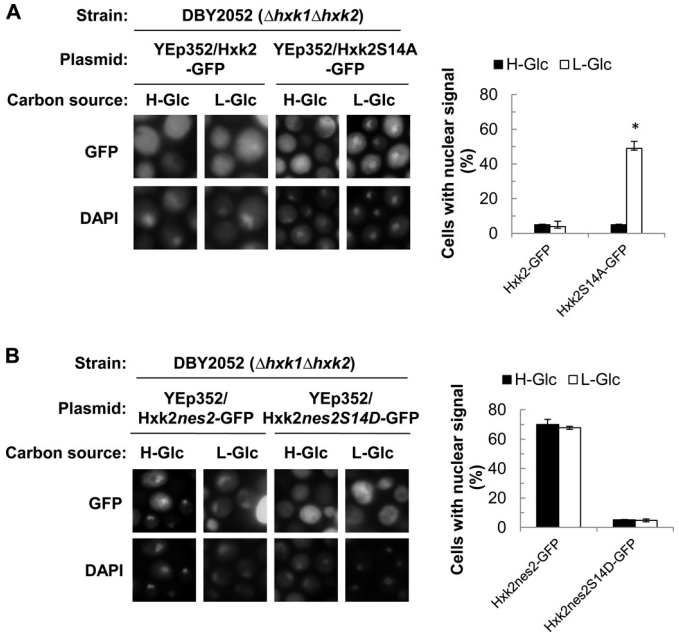

FIGURE 2. **Localization of Hxk2S14A and Hxk2S14D in Δ*hxk1*Δ*hxk2* yeast cells.** The DBY2052 (Δ*hxk1*Δ*hxk2*) mutant strain was transformed with plasmids YEp352/Hxk2-GFP, YEp352/Hxk2S14A-GFP (*A*), YEp352/Hxk2nes2-GFP, and YEp352/Hxk2*nes2*S14D-GFP (*B*) plasmids. Transformed cells were grown in high glucose synthetic medium (*H-Glc*) until an $A_{600\ nm}$ of 1.0 was reached and then transferred to low glucose synthetic medium (*L-Glc*) for 60 min. The cells were visualized by fluorescence microscopy; DAPI staining revealed nuclear DNA. The nuclear localization of fluorescent reporter proteins was determined in at least 100 cells in three independent experiments. Means and S.D. (*error bars*) are shown for at least three independent experiments. *, statistically significant differences of Hxk2S14A *versus* Hxk2 in low glucose conditions, $p < 0.01$.

nuclear import and export of Hxk2 are affected by its phosphorylation at serine 14.

Hxk2 Phosphorylation Affects Its Nucleocytoplasmic Distribution—To investigate the physiological importance of Hxk2 phosphorylation, we used the non-phosphorylatable (Hxk2S14A) or phosphomimetic (Hxk2S14D) form of Hxk2 fused to the GFP protein to examine its nucleocytoplasmic distribution in both high and low glucose conditions. As has been noted before (13), the fluorescence signals elicited by the Hxk2S14A-GFP mutant cells show a mostly cytoplasmic distribution in high glucose conditions. However, 50% of cells showed nuclear accumulation of Hxk2S14A-GFP after a shift to low glucose conditions (Fig. 2*A*). The increase of nuclear signal is probably the consequence of a failure in Hxk2-Xpo1 interaction. To analyze the nucleocytoplasmic distribution of the phosphomimetic Hxk2S14D-GFP protein, we took advantage of the fact that the Hxk2*nes2* variant of Hxk2 is strongly export-defective with an export defect in ~72% of the cells. The nuclear accumulation of Hxk2*nes2* was strong in both high and low glucose conditions (13). Thus, we constructed an Hxk2*nes2*S14D mutant protein to investigate the presence of defects in its nuclear accumulation by fluorescence microscopy. As can be seen in Fig. 2*B*, Hxk2*nes2*S14D showed a loss of nuclear accumulation in Δ*hxk1*Δ*hxk2* mutant cells, indicating that serine 14 phosphorylation inhibits the import of Hxk2 from the cytoplasm to the nucleus. The decrease of nuclear signal is probably the consequence of a failure in phosphomimetic Hxk2S14D-Kap60 interaction.

The Hxk2S14D Mutant Exhibits an Increased Electronegative Patch on the Protein Surface—Structural comparison of Hxk2S14D, Hxk2S14A, and the wild-type (Hxk2) proteins revealed no significant difference in their interlobe orientations. Taking into account that these structures are based on modeling and not on structural data, when we analyzed the serine 14 area in the Hxk2 or Hxk2S14A structure, we found that the amino acid is in a hydrophobic patch in close proximity to the NLS1 motif (Lys-6 to Lys-12), which is also located in a hydrophobic patch in an extended configuration (Fig. 3*A*). However, in the Hxk2S14D mutant structure, aspartic acid 14 makes the NLS region more electronegative and hydrophilic; simultaneously, the NLS1 motif acquired a more folded configuration; and the N-terminal valine residue approached the protein surface between the NES1 and NES2 motifs (Fig. 3*B*). The distance between the Cα of aspartic acid 17 and histidine 2 in the Hxk2 (WT) or Hxk2S14A structures was 35.84 Å, whereas in the Hxk2S14D structure, the distance between the Cα of aspartic acid 17 and Cα of histidine 2 was 10.68 Å. Thus, a change in the hydrophobicity and in the charge of the NLS1 region by mutation of serine 14 to aspartic acid may promote a change in the NLS1 motif folding that brings aspartic acid 17 closer to histidine 2 (Fig. 3*C*). Moreover, because the distance between Cα of lysine 6 (in the NLS1 motif) and valine 375 on the surface of the protein is 29.27 Å in the Hxk2S14A structure, whereas in the Hxk2S14D structure, the distance between lysine 6 and valine 375 is 12.22 Å, we suggest that the NLS1 motif of Hxk2 is also closer to the protein surface in the phosphorylated state (Fig. 3*C*). These results indicate that the phosphorylation state of serine 14 is an important factor for structural conformation of the NLS1 motif of Hxk2, suggesting a correlation between the phosphorylation state of Hxk2 and the proper regulation of its interaction with the α-importin (Kap60). The structural change in the N-terminal region of Hxk2 induced by the phosphorylation state of serine 14 also affects the NES1 and NES2 regions. The distances between the Cα of valine 1 in the Hxk2 (WT) or Hxk2S14A structures and the Cα of leucine-22 (NES1) and methionine 318 (NES2) were 38.31 and 47.22 Å, respectively, whereas in the Hxk2S14D structure, the distance between the Cα of valine 1 and the Cα of leucine 22 (NES1) and methionine 318 (NES2) was 17.18 and 22.6 Å, respectively (Fig. 3*D*). These data also indicate that the phosphorylation state of serine 14 is important for the structural conformation of the NES region, suggesting that the folding of the N-terminal region of Hxk2 between the NES1 and NES2 motifs is important for proper interaction between Hxk2 and Xpo1.

Snf1 Regulates the Nucleocytoplasmic Distribution of Hxk2—In low glucose conditions, Hxk2 is phosphorylated at serine 14 (11). How this phosphorylation, catalyzed by a still unknown kinase, may affect nucleocytoplasmic shuttling of Hxk2 and glucose signaling is unknown at the moment (8, 12, 38). Because the phosphorylated serine 14 residue of the Hxk2 protein lies within consensus phosphorylation sites of PKA and Snf1 protein kinases, we first investigated whether Hxk2 nucleocytoplasmic distribution was affected in Δ*tpk1*, Δ*tpk2*, Δ*tpk3*, and Δ*snf1* mutants. To test this, we analyzed the subcellular localization of Hxk2-GFP by fluorescence microscopy (Fig. 4*A*).

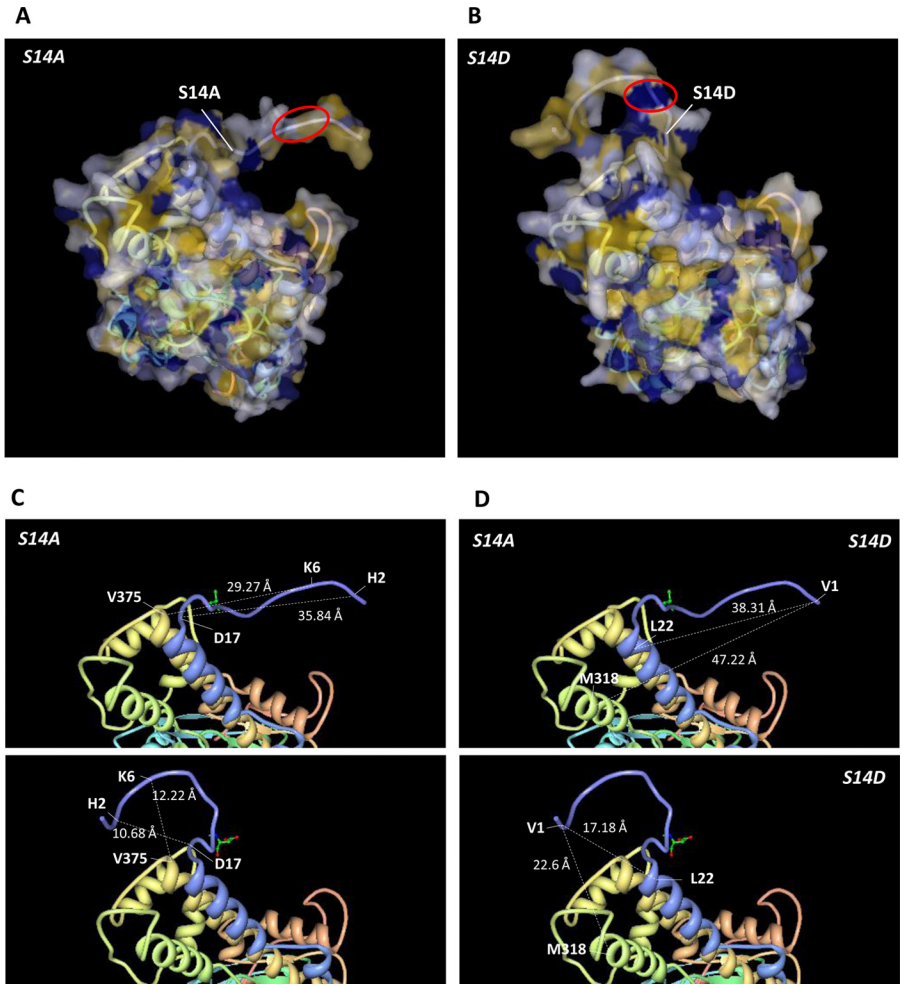

FIGURE 3. **Structural analysis of the Hxk2 NLS region.** Electrostatic surface presentations of Hxk2S14A (*A*) and Hxk2S14D (*B*) are shown. The NLS region (*circled*) displays increased electronegativity due to the S14D mutation, which could have an effect on Xpo1 recognition. *C*, the distances between the Cα of different residues (His-2 to Asp-17 and Lys-6 to Val-375) from Hxk2S14A and Hxk2S14D are shown; they suggest a significant conformational change in the NLS region. *D*, the distances between the Cα of different residues (Val-1 to Leu-22 and Val-1 to Met-318) from Hxk2S14A and Hxk2S14D are shown; they suggest that the conformational change in the NLS region introduces Val-1 between the two NES sequences.

The fluorescence signals elicited by wild-type and Δ*tpk1*, Δ*tpk2*, and Δ*tpk3* mutant cells expressing the Hxk2-GFP fusion protein show a mostly cytoplasmic distribution in both high and low glucose conditions. Identical results were obtained by using the double disruption mutant strains Δ*tpk1*Δ*tpk2*, Δ*tpk1*Δ*tpk3*, and Δ*tpk2*Δ*tpk3*, each lacking two of the three possible catalytic subunits of PKA (data not shown). However, because the PKA catalytic subunits are redundant for many of their functions and the Δ*tpk* double mutants retain PKA activity, it is difficult from these results to conclude that PKA plays no role in Hxk2 serine 14 phosphorylation.

Although strains expressing only one of the *TPK* genes grow normally, a triple null mutant (Δ*tpk1*Δ*tpk2*Δ*tpk3*) is not viable (39). PKA is needed to counteract the negative effect of the protein kinase Yak1 on yeast growth (40, 41). In the presence of PKA, the transcription of the *YAK1* gene is reduced, and Yak1 levels remain low (42). This explains why strains lacking Yak1 no longer require PKA for growth (43). Thus, to determine whether any PKA catalytic subunit plays a role in Hxk2 serine 14 phosphorylation, we used a strain lacking PKA and carrying the suppressor mutation Δ*yak1*. The utilization of this triple mutant strain avoids redundant functions of the catalytic subunits of PKA. Moreover, in this strain, glucose induction of genes related to glucose metabolism or glucose repression of genes involved in the utilization of carbon sources other than glucose can proceed in the absence of PKA (44).

We found that the subcellular localization of Hxk2-GFP shows a mostly cytoplasmic distribution in both high and low glucose conditions (Fig. 4*B*). Moreover, we examined whether the absence of Tpk subunits affects the Hxk2 interaction with Kap60 and Xpo1 by GST pulldown assays in both high and low glucose conditions. As expected, the absence of Tpk subunits resulted in an interaction of Hxk2 with Kap60 (Fig. 5*A*) and Xpo1 (Fig. 5*B*) identical to that observed in a wild-type strain under both high and low glucose conditions.

These results suggest that Hxk2-GFP can enter and exit the nucleus in the absence of PKA catalytic subunits as it does in a wild-type cell. Therefore, Tpk1, Tpk2, and Tpk3 do not seem to be implicated in Hxk2 nucleocytoplasmic distribution regulation.

However, the Δ*snf1* mutant cells showed nuclear accumulation of Hxk2-GFP after a shift to low glucose conditions (Fig.

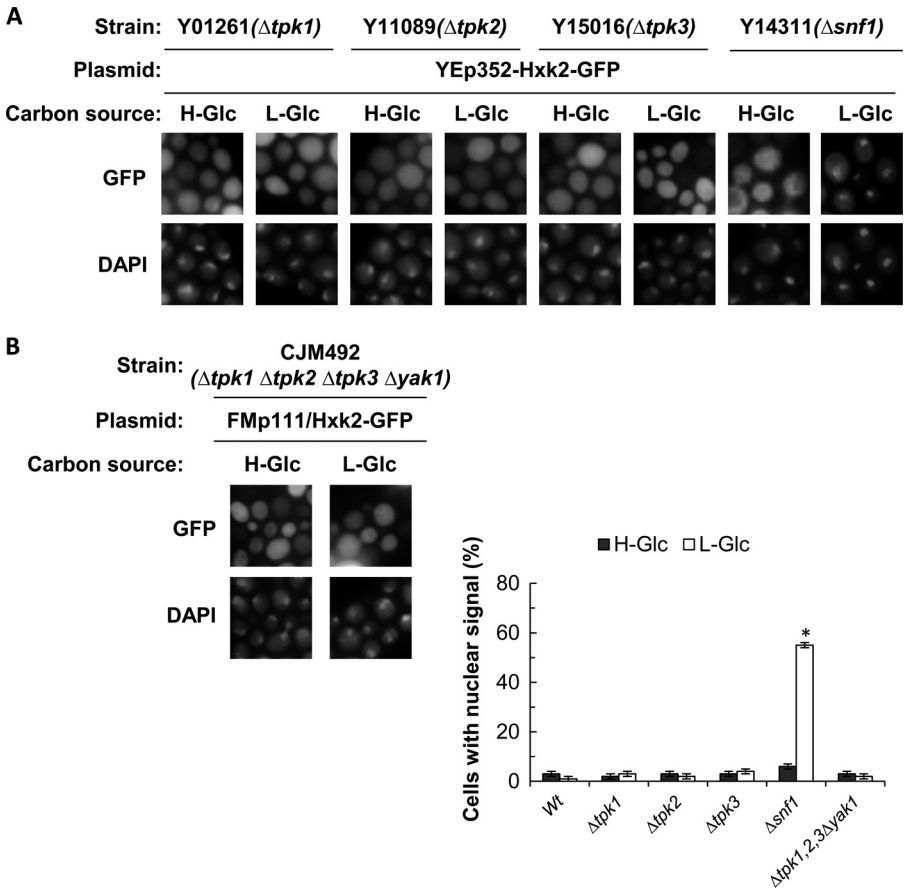

FIGURE 4. **Localization of Hxk2-GFP in *PKA and* Δ*snf1* mutant cells.** The Y01261, Y11089, Y15016, and Y14311 strains expressing Hxk2-GFP from plasmid YEp352/Hxk2-GFP were grown in high glucose synthetic medium (*H-Glc*) until an $A_{600\,nm}$ of 1.0 was reached and then transferred to low glucose synthetic medium (*L-Glc*) for 60 min. Cells were stained with DAPI and imaged for GFP and DAPI fluorescence. The nuclear localization of Hxk2-GFP protein was determined in at least 100 cells per growth condition. *, statistically significant differences of wild type *versus* Δ*snf1* in low glucose conditions, $p < 0.01$. *Error bars* represent S.E.

4*A*). Our data show that in the absence of the Snf1 protein kinase 55% of cells expressing Hxk2-GFP showed nuclear accumulation of Hxk2 similar to that found in an Hxk2S14A strain (13). Because an alanine substitution at serine 14 of Hxk2 (Hxk2S14A) inhibits its interaction with Xpo1 under low glucose conditions, the increase of nuclear signal in Δ*snf1* mutant cells is probably the consequence of a failure in serine 14 phosphorylation of Hxk2 and thus in Hxk2-Xpo1 interaction.

Moreover, because Hxk2 exists as a monomer and a homodimer (12, 45) and its nuclear import takes place under high glucose conditions, we cannot exclude that protein dimerization was required for protein nuclear importation. Recently, the Tda1 protein has been described as the necessary kinase for dissociation of the Hxk2 dimer *in vitro* upon serine 14 phosphorylation (46). Thus, we investigated whether the Hxk2 nucleocytoplasmic distribution is affected in Δ*tda1* mutant cells. To test this, we analyzed the subcellular localization of Hxk2-GFP by fluorescence microscopy and the Hxk2 interaction with Kap60 and Xpo1 karyopherins in Δ*tda1* mutant cells. These experiments gave results identical to those obtained with the wild-type strain (Fig. 6, *A* and *B*). Thus, our data strongly suggest that there is no regulatory significance to Hxk2 dimerization in the nucleocytoplasmic shuttling of Hxk2.

Snf1 and Reg1 Regulate the Hxk2 Interaction with Kap60 and Xpo1—We have shown previously that the nucleocytoplasmic transport of Hxk2 is executed by its glucose-regulated interaction with α-importin (Kap60) and the exportin Xpo1 (13, 20). Because Kap60 and Xpo1 preferentially recognize Hxk2 under high and low glucose conditions, respectively, we examined whether the absence of Snf1 (Δ*snf1*) and Reg1 (Δ*reg1*) affects the endogenous Hxk2 interaction with Kap60 and Xpo1 in both high and low glucose conditions by GST pulldown assays. As expected, the absence of Snf1 resulted in an enhanced interaction of Hxk2 with Kap60 and a loss of Hxk2 interaction with Xpo1 under low glucose conditions (Fig. 7*A*). These results clearly suggest the involvement of Snf1-mediated Hxk2 phosphorylation in the interaction with Kap60 and Xpo1 and thereby in the nucleocytoplasmic distribution of Hxk2.

To provide independent verification that the activated Snf1 protein associates with Hxk2 *in vivo*, we used an immunoprecipitation assay in cells expressing Snf1-HA (used as control) or Snf1T210A-HA as the only Snf1 protein. Because Snf1 activation is regulated in response to glucose concentrations, extracts were prepared from cells grown under high and low glucose conditions. Cell extracts from these strains were immunoprecipitated with anti-Hxk2 antibodies. As shown in Fig. 7*B*, con-

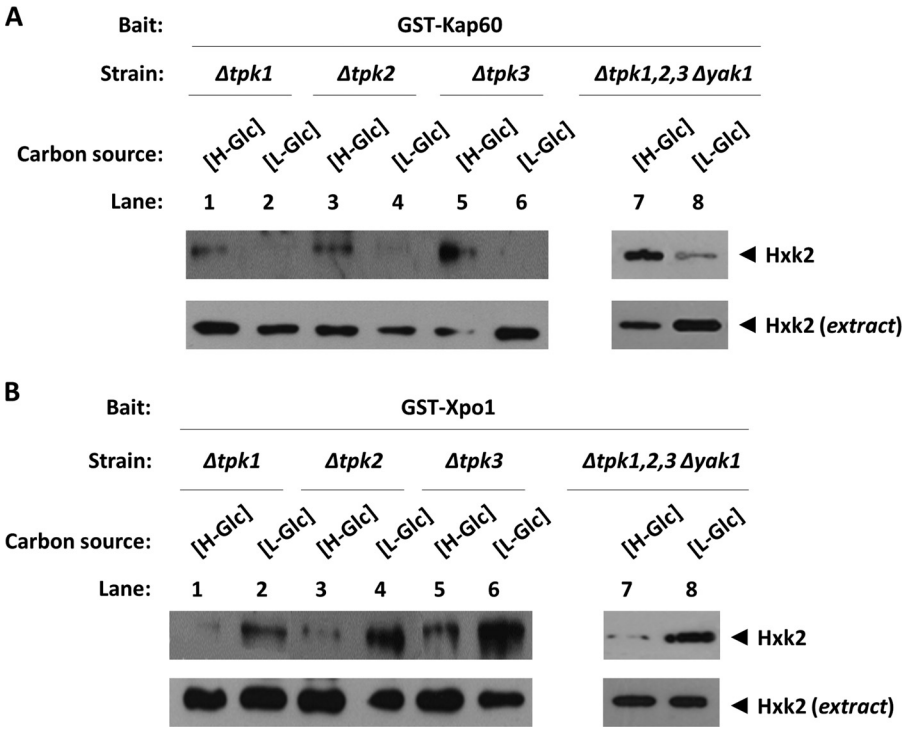

FIGURE 5. **GST pulldown assays of the interaction of Kap60 and Xpo1 with Hxk2 in PKA mutant cells.** The GST-Kap60 and GST-Xpo1 fusion proteins were purified on glutathione-Sepharose columns. Equal amounts of GST-Kap60 (*A*) and GST-Xpo1 (*B*) were incubated with cell extracts from the Y01261, Y11089, and Y15016 strains. The yeast strains were grown in YEPD medium until an $A_{600\ nm}$ of 0.8 was reached and then shifted to low glucose (*L-Glc*) conditions for 1 h. After exhaustive washing, the proteins were separated by 12% SDS-PAGE, and retained Hxk2 was visualized on a Western blot with polyclonal anti-Hxk2 antibody. For the control samples, GST protein was also incubated with the high glucose (*H-Glc*) cell extracts, but no signals were detected (data not shown). The level of Hxk2 present in the different extracts was determined by Western blot using anti-Hxk2 antibody.

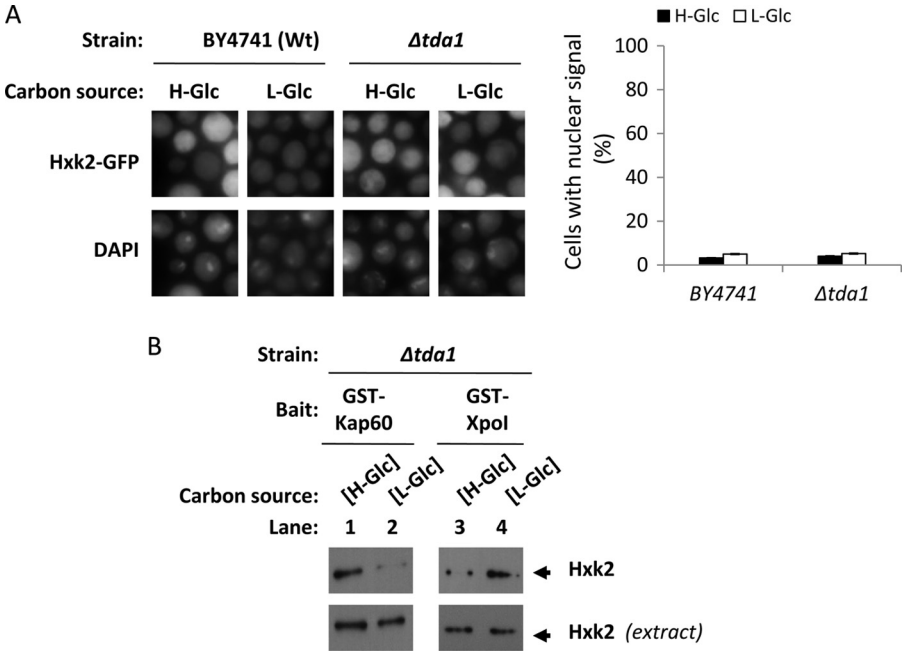

FIGURE 6. *A*, localization and interaction of Hxk2 with Kap60 and Xpo1 in Δ*tda1* mutant cells. The Y10878 and BY4741 strains expressing Hxk2-GFP from plasmid YEp352/Hxk2-GFP were grown in high glucose synthetic medium (*H-Glc*) until an $A_{600\ nm}$ of 1.0 was reached and then transferred to low glucose synthetic medium (*L-Glc*) for 60 min. Cells were stained with DAPI and imaged for GFP and DAPI fluorescence. The nuclear localization of Hxk2-GFP protein was determined in at least 100 cells per growth condition. *B*, GST pulldown assays of the interaction of Kap60 and Xpo1 with Hxk2 in Δ*tda1* mutant cells. The GST-Kap60 and GST-Xpo1 fusion proteins were purified on glutathione-Sepharose columns. Equal amounts of GST-Kap60 and GST-Xpo1 were incubated with cell extracts from the Y10878 mutant strain. The yeast strain was grown in YEPD medium until an $A_{600\ nm}$ of 0.8 was reached and then shifted to low glucose conditions for 1 h. After exhaustive washing, the proteins were separated by 12% SDS-PAGE, and retained Hxk2 was visualized on a Western blot with polyclonal anti-Hxk2 antibody (*lanes 1–4*). For the control samples, GST protein was also incubated with the high and low glucose cell extracts, but no signals were detected (data not shown). The level of Hxk2 present in the different extracts used was determined by Western blot using anti-Hxk2 antibody. The Western blots shown are representative of results obtained from three independent experiments. *Error bars* represent S.E.

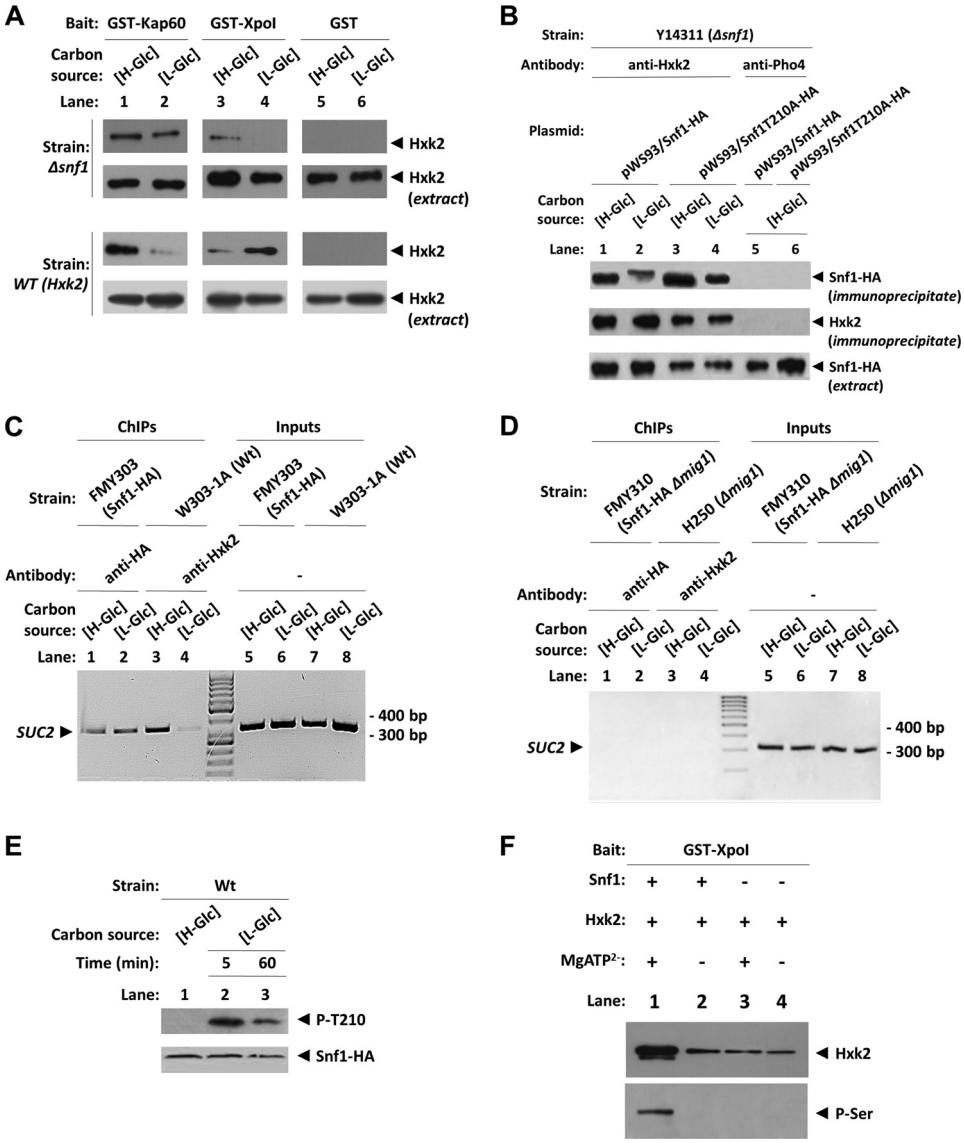

FIGURE 7. **Snf1 directly phosphorylates serine 14 of Hxk2.** *A*, GST pulldown assays of the interaction of Kap60 and Xpo1 with Hxk2 in *snf1* mutant cells. The GST-Kap60 and GST-Xpo1 fusion proteins were purified using glutathione-Sepharose columns. Equal amounts of GST-Kap60 and GST-Xpo1 were incubated with cell extracts from the Y14311 (*snf1*) mutant strain. The yeast strain was grown in YEPD medium until an $A_{600\ nm}$ of 0.8 was reached and then shifted to low (*L-Glc*) glucose conditions for 1 h. After exhaustive washing, the proteins were separated by 12% SDS-PAGE, and retained Hxk2 was visualized on a Western blot with polyclonal anti-Hxk2 antibody (*lanes 1–4*). For the control samples, GST protein was also incubated with the high and low glucose cell extracts, but no signals were detected (*lanes 5* and *6*). The level of Hxk2 present in the different extracts used in *A* was determined by Western blot using anti-Hxk2 antibody. *B*, *in vivo* co-immunoprecipitation of Snf1 with Hxk2. The Y14311 (*snf1*) strain expressing Snf1-HA or Snf1T210A-HA from plasmid pWS93/Snf1-HA or pWS93/Snf1T210A-HA, respectively, was grown in high glucose synthetic medium (*H-Glc*) until an $A_{600\ nm}$ of 1.0 was reached and then transferred to low glucose synthetic medium for 60 min. The cell extracts were immunoprecipitated with a polyclonal anti-Hxk2 antibody (*lanes 1–4*) or a polyclonal antibody to Pho4 (*lanes 5* and *6*). Immunoprecipitates were separated by 12% SDS-PAGE, and co-precipitated Snf1 was visualized on a Western blot with monoclonal anti-HA antibody. The level of immunoprecipitated Hxk2 in the blotted samples was determined by using anti-Hxk2 antibody. The level of Snf1-HA or Snf1T210A-HA present in the different extracts was determined by Western blot using anti-HA antibody. *C*, *in vivo* binding of Snf1 to the MIG1 element of the *SUC2* promoter. FMY303 cells expressing an HA-tagged version of Snf1 and wild-type cells were grown in high and low glucose conditions. Cell extracts were prepared and immunoprecipitated with anti-HA or anti-Hxk2 antibodies, and the DNA fragments were amplified by PCR using the combination of oligonucleotides as described under "Experimental Procedures." The amplified fragments were resolved by agarose gel electrophoresis. The migration of standard markers is indicated on the *right-hand side*. A representative ChIP assay from three independent experiments is shown. *D*, *in vivo* binding of Snf1 to the MIG1 element of the *SUC2* promoter is Mig1-dependent. FMY310 (Snf1-HA *mig1*) cells expressing an HA-tagged version of Snf1 and H250 (*mig1*) cells were grown in high and low glucose conditions. Cell extracts were prepared and immunoprecipitated with anti-HA or anti-Hxk2 antibodies, and the DNA fragments were amplified by PCR using the combination of oligonucleotides as described under "Experimental Procedures." The amplified fragments were resolved by agarose gel electrophoresis. The migration of standard markers is indicated on the *right-hand side*. A representative ChIP assay from three independent experiments is shown. *E*, phosphorylation at threonine 210 of Snf1. Phosphorylation of threonine 210 of Snf1 protein kinase was detected by Western blot using antibody to phosphothreonine 172 of AMP-activated protein kinase that also specifically recognizes phosphothreonine 210 (*P-T210*) of Snf1. The total amount of Snf1 protein in the different extracts was determined by using anti-HA antibody. *F*, *in vitro* kinase assay. Identical amounts of Snf1 and Hxk2 affinity-purified proteins were incubated in the presence of $MgATP^{2-}$. The GST-Xpo1 fusion protein coupled to glutathione-Sepharose was incubated with the assay mixture for 1 h at 4 °C in PBS buffer. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 μl of sample loading buffer, and analyzed by SDS-PAGE followed by Western blot using anti-Hxk2 and anti-phosphoserine (*P-Ser*) antibodies. The Western blot shown is representative of results obtained from four independent experiments.

sistent levels of Hxk2 protein were detected by Western blotting in the immunoprecipitates when anti-Hxk2 antibody was used. When an anti-Pho4 antibody was used to detect unspecific immunoprecipitation, no signals were observed. Similar amounts of the Snf1-HA and Snf1T210A-HA proteins were also detected in the different protein extracts used for immunoblot analysis with an anti-HA antibody. Following immune precipitation with anti-Hxk2 antibodies, the Snf1 and Snf1T210A proteins were detected in a complex with Hxk2 from both glucose-starved cells (*lanes 2* and *4*) and glucose-rich medium-grown cells (*lanes 1* and *3*). In accordance with previous results, we conclude that Snf1 associates with Hxk2 *in vivo* and that the association is independent of the glucose levels in the culture medium (5). However, our results also suggest that Snf1 activation by threonine 210 phosphorylation is not required for the Snf1-Hxk2 association. Previous reports have demonstrated that Hxk2 participates in the *SUC2*-Mig1 repressor complex by interaction with Mig1 protein and not by direct binding to DNA (5, 7, 10). As Snf1 and Hxk2 interact in both high and low glucose conditions, we investigated the biological significance of this interaction by determining whether Snf1 localized in the *SUC2*-Mig1 repressor complex. To test this hypothesis, we used ChIP assays. Our results show that in cells grown in high glucose medium both Snf1 and Hxk2 proteins were recruited to a DNA fragment of the *SUC2* promoter containing the Mig1 binding site (Fig. 7*C*, *lanes 1* and *3*). Conversely, in low glucose medium, although Snf1 remains as a component of the complex, Hxk2 binding to the *SUC2*-Mig1 complex was abolished (Fig. 7*C*, *lanes 2* and *4*). Binding of Snf1-HA and Hxk2 to the Mig1 site of the *SUC2* promoter was dependent on the presence of Mig1 because in Δ*mig1* cells no binding of Snf1-HA or Hxk2 to the *SUC2* promoter was observed under any growth conditions (Fig. 7*D*). No DNA amplification was observed when we used cells with untagged Snf1 or oligonucleotides for amplification of a DNA fragment from *ACT1* promoter (data not shown).

To confirm these results, we examined the phosphorylation of threonine 210 of Snf1 protein kinase in cells exposed to high and low glucose conditions. Thus, we carried out an immunoblot analysis using an antibody against phosphothreonine 172 of AMP-activated protein kinase that also specifically recognizes phosphothreonine 210 of Snf1 (48, 49). In accordance with previous results (16, 50), the threonine 210 residue of Snf1 kinase was phosphorylated in a transitory manner when cells were shifted from high to low glucose conditions (Fig. 7*E*). The fact that the Snf1-Hxk2 interaction does not depend on the degree of Snf1 phosphorylation suggests that in this system the phosphorylation status of the Snf1 activation loop might be important for activation of the Snf1 catalytic activity (49, 50) but not for the Snf1-Hxk2 interaction.

The interaction of Hxk2 with Snf1 prompted us to investigate whether Hxk2 could be directly phosphorylated by Snf1. We performed an *in vitro* assay in which recombinant Hxk2 produced in bacteria was used as a substrate for the Snf1 complex purified from yeast grown under low glucose conditions. Because Xpo1 preferentially recognizes Hxk2 phosphorylated at serine 14, we used this criterion to determine the phosphorylation state of serine 14 in the different samples. As can be seen

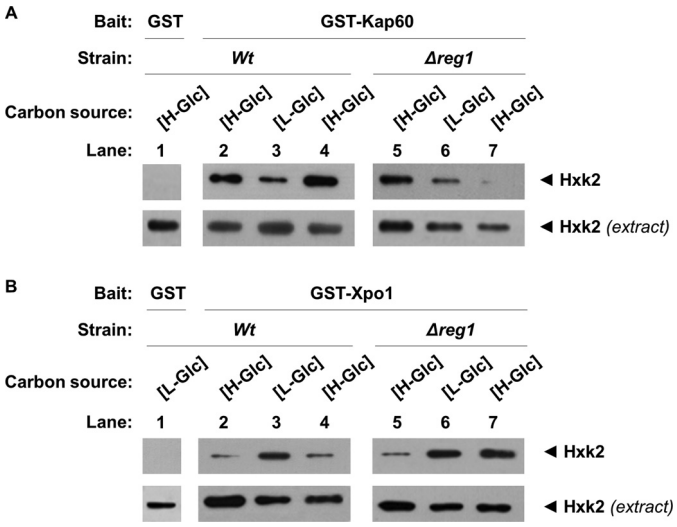

FIGURE 8. **GST pulldown assays of the interaction of Kap60 and Xpo1 with Hxk2 in Δ*reg1* mutant cells.** The GST-Kap60 and GST-Xpo1 fusion proteins were purified on glutathione-Sepharose columns. Equal amounts of GST-Kap60 (*A*) and GST-Xpo1 (*B*) were incubated with cell extracts from the BY4741 (WT) and Y13967 strains. The yeast strains were grown in YEPD medium until an $A_{600\ nm}$ of 0.8 was reached and then shifted to low glucose (*L-Glc*) conditions for 1 h following a shift to high glucose (*H-Glc*) conditions for 1 h. After exhaustive washing, the proteins were separated by 12% SDS-PAGE, and retained Hxk2 was visualized on a Western blot with polyclonal anti-Hxk2 antibody. For the control samples, GST protein was also incubated with the high and low glucose cell extracts, respectively, but no signals were detected (*lane 1*). The level of Hxk2 present in the different extracts used in Fig. 8, *A* and *B*, was determined by Western blot using anti-Hxk2 antibody. The Western blots shown are representative of results obtained from four independent experiments.

in Fig. 7*F*, purified Snf1 enzyme did promote phosphorylation of the Hxk2 protein at residue serine 14 because a strong increase in interaction between Hxk2 and Xpo1 was detected (*lane 1*). In control experiments, the bacterially produced Hxk2 shows a basal signal similar to that found in the absence of Snf1 or $MgATP^{2-}$ (*lanes 2–4*). To directly assess the presence of phosphoserine in Hxk2, we carried out an immunoblot analysis with an anti-phosphoserine antibody. This antibody detects phosphoserine Hxk2 but not Hxk2, indicating that phosphoserine Hxk2 has been produced in the reaction mixture of our assay (*lane 1*). We conclude that the Snf1 protein kinase directly promotes phosphorylation of Hxk2 at serine 14 *in vitro*.

Genetic and biochemical studies have shown that Reg1 associates with Hxk2, and this association stimulates the phosphorylation of Reg1 by Snf1 (51). The Reg1 protein is one of the regulatory subunits of type 1 protein phosphatase Glc7 (52). The fact that Hxk2 interacts with Reg1 suggests that the Glc7-Reg1 complex could act in opposition to the Snf1 kinase by promoting the dephosphorylation of Hxk2 serine 14 under high glucose conditions. To explore this further, cells were grown to midlog phase in YEPD medium and then shifted to low glucose conditions for 1 h to induce Hxk2 phosphorylation at serine 14 by Snf1 kinase. Finally, cells with phosphorylated Hxk2 were shifted to high glucose conditions to assay whether the absence of Reg1 (Δ*reg1*) affected the endogenous Hxk2 interaction with Kap60 and Xpo1 in both high and low glucose conditions by GST pulldown experiments. We found that deletion of *REG1* effectively suppressed the Hxk2-Kap60 interaction after a shift of cells from low to high glucose conditions (Fig. 8*A*). In con-

trast, the absence of Reg1 resulted in an increased Hxk2 interaction with Xpo1 under high glucose conditions (Fig. 8*B*). These results clearly suggest the involvement of Reg1-mediated Hxk2 dephosphorylation in the interaction with Kap60 and Xpo1 and therefore in the nucleocytoplasmic distribution of Hxk2.

DISCUSSION

Many proteins shuttle continuously back and forth between the nucleus and cytoplasm of eukaryotic cells. The nucleocytoplasmic distribution of some of these proteins, particularly those involved in signaling, is known to be regulated. Most of the proteins undergo regulated binding and release from the receptors by masking and unmasking of NLSs and NESs. Both the release from the binding sites or exposure of the binding sites can be brought about by monomer-dimer equilibrium (24), phosphorylation-dephosphorylation (53, 54), or in some cases proteolysis.

The Hxk2 protein shuttles between the nucleus and the cytoplasm in response to glucose availability. Recently, the mechanisms of nuclear Hxk2 export (13) and import (20) have been reported, but the Hxk2 nucleocytoplasmic shuttling regulation remained to be established. One important feature to understand the regulation of the nucleocytoplasmic shuttling of Hxk2 is that Hxk2 is a phosphoprotein. Moreover, Hxk2 exists *in vivo* in a dimer-monomer equilibrium that is affected by phosphorylation. Only the monomeric form is phosphorylated, whereas the dimer is not. The reversible phosphorylation of Hxk2 is carbon source-dependent, being more extensive in low glucose conditions (12, 46). These observations prompted us to examine whether phosphorylation and dephosphorylation are involved in the nuclear export and import of Hxk2.

In this study, we present several lines of evidence that suggest that the shuttling back and forth between the nucleus and the cytoplasm of Hxk2 is regulated by phosphorylation and dephosphorylation at serine 14 mediated by Snf1 kinase and the Reg1-dependent Glc7 protein phosphatase, respectively. We show that the Snf1 kinase is constitutively associated with Hxk2 both at high and low glucose conditions and that Hxk2 accumulates in the nucleus upon *SNF1* gene disruption. We also report that both Snf1 and Hxk2 interact in a Mig1-dependent manner in a cluster with DNA fragments containing the MIG1 binding site of the *SUC2* promoter. Taking all these results together, we suggest that Snf1 forms a complex in the nucleus with Mig1 and Hxk2 and that this complex is involved in the glucose-regulated expression of the *SUC2* gene. This complex dissociates and exits the nucleus under conditions of Snf1 activation; the presence of Xpo1 (13) and Msn5 (55) are necessary for the nuclear exit of Hxk2 and Mig1, respectively. Thus, the nuclear export of Hxk2 requires phosphorylation of serine 14. Failure to phosphorylate this residue due to mutation to alanine causes a significant fraction of Hxk2 to be retained in the nucleus. These observations also suggest that it is the phosphorylated form of Hxk2 that is exported to the cytoplasm. Additionally, we demonstrate by *in vitro* experiments using purified proteins that Snf1 kinase directly phosphorylates Hxk2 at serine 14. It has also been described that Reg1 and Hxk2 are associated (51). In this work, we demonstrate that the protein phosphatase Glc7-Reg1, following its activation by high glucose conditions, dephosphorylates Hxk2 at serine 14 and thus appears to play an essential role in Hxk2 nuclear import as the dephosphorylated Hxk2 binds to Kap60 to form the import complex.

The nucleocytoplasmic shuttling of Hxk2 described above is analogous to that observed for several transcription factors (29, 37, 56, 57). These transcription factors are present in yeast cells in an inactive state in the cytoplasm as phosphoproteins and appear to depend on nuclear import for their function *in vivo*. In response to signal-mediated elevated glucose levels in the medium, activated phosphatases, like Glc7-Reg1, dephosphorylate critical serine residues in these cytosolic transcription factors, allowing them to be imported to the nucleus for targeted gene activation or inhibition. Cessation of glucose signaling results in phosphorylation of these transcription factors in the nucleus by nuclear activated protein kinases, like Snf1 kinase, and allows these proteins to be exported to the cytosol as phosphoproteins (58). However, it should be emphasized that the present work is the first to show nucleocytoplasmic shuttling regulation of a metabolic enzyme that functions as a glycolytic enzyme in the cytoplasm and as a regulator of gene transcription of several Mig1-regulated genes in the nucleus. In this sense, it has been shown previously that the nuclear activity of Hxk2 is important for the activation of the Mig1 transcription factor necessary for relevant gene repression (4, 5). Therefore, nucleocytoplasmic translocation of Hxk2 is essential for the repression or induction of many Mig1-dependent processes.

An important question that still awaits investigation is why strong differences exist in the amount of Hxk2 distributed between the nucleus and the cytoplasm in both high and low glucose conditions. This question arises because Hxk2 is predominantly localized in the cytoplasm in both high and low glucose conditions. However, a small fraction of total Hxk2 is dephosphorylated by Glc7-Reg1 phosphatase, which promotes an increase in binding affinity to Kap60 and a reduction in binding affinity to Xpo1, favoring Hxk2 nuclear accumulation. Phosphorylation of nuclear Hxk2 at serine 14, a residue immediately adjacent to the NLS, by Snf1 kinase promotes an increase in binding affinity to Xpo1 and a decrease in binding affinity to Kap60, producing the net exit of nuclear Hxk2 to the cytoplasm. These changes in Hxk2 binding affinity for its karyopherins correlate with important structural changes in the N-terminal region of the protein. Hxk2 phosphorylation at serine 14 forms an acidic patch in the NLS region that induces binding of Hxk2 to Xpo1 and consequently allows nuclear export through the nuclear pore. Hxk2 dephosphorylation at serine 14 by Glc7-Reg1 protein phosphatase forms an extended hydrophobic patch in the NLS1 motif region that induces binding of Hxk2 to Kap60 and consequently allows nuclear import through the nuclear pore.

In summary, we have found that the Hxk2 is phosphorylated and dephosphorylated at serine 14 by Snf1 kinase and Glc7-Reg1 phosphatase, respectively, demonstrating for the first time a new cross-talk between them that controls nucleocytoplasmic shuttling of Hxk2. Regulating the subcellular distribu-

tion of Hxk2 represents a novel physiological function mediated by the Snf1 and Glc7-Reg1 proteins.

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